

Protein thiol oxidoreductas and allergic airways disease

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Valorisation

This thesis is focused on the identification of redox mechanisms, notably the role of thiol oxidoreductases and protein thiol oxidation in human allergic asthma as well as murine models of allergic airways disease.

Application: a role for glutaredoxin-1 and S-glutathionylation in epithelial cell apoptosis and fibrosis

Additional studies from our laboratory have demonstrated the S-glutathionylation/glutaredoxin redox axis is an important module in the pathogenesis of multiple lung diseases. *Pseudomonas aeruginosa*, a well-studied ubiquitous pathogen commonly found in the lungs of cystic fibrosis and immunocompromised patients [1], also causes significant morbidity and mortality in critically ill ventilated patients [1]. Furthermore, activation of the death receptor, Fas, in the airway epithelium is a critical component of *P.Aeruginosa* clearance following infection [2, 3]. *P.Aeruginosa* produces numerous redox active compounds [4-6], and our group has demonstrated that S-glutathionylation of Fas enhances formation of the death-inducing signaling complex and accelerates apoptosis in epithelial cells and promoted clearance of *Pseudomonas aeruginosa*. Conversely, overexpression of Grx1 resulted in deglutathionylation of Fas, decreased FasL-induced apoptosis in lung epithelial cells, and decreased clearance of *Pseudomonas aeruginosa* [7], highlighting the importance of the precise balance of Fas-SSG and Grx1 [8].

Activation of Fas has also been shown to be causal in the pathogenesis of bleomycin (BLM)-induced lung remodeling, in part by increasing epithelial cell apoptosis. Increased loss of epithelial cells is currently believed to play an important role in the pathogenesis of pulmonary fibrosis. Additional studies from our laboratory revealed increased protein S-glutathionylation within the bronchiolar epithelium [9], following oropharyngeal aspiration of bleomycin. Changes in glutathione content have also been previously reported in both rodent models of fibrosis and patients with IPF [10, 11] however, the extent to which protein S-glutathionylation, including Fas-SSG, is altered in these settings and contributes to disease pathogenesis, remains unknown. Investigation by our laboratory discovered a latent pool of Fas localized to the endoplasmic reticulum (ER) and upon activation of surface Fas, this separate pool was S-glutathionylated and shuttled to the surface, suggesting that FasL induces rapid changes in the redox status of the ER [12]. As previously mentioned, intramolecular disulfide (S-S) bond formation is catalyzed by protein disulfide isomerases (PDIs) [13-15], and stimulation with FasL resulted

in a strong association between ERp57 and Fas. PDIs such as ERp57 also produce H₂O₂ [16, 17], which is thought to promote protein S-glutathionylation via formation of a sulfenic acid intermediate. Glutathione S-transferases are classically known as phase II detoxifying enzymes that catalyze GSH conjugation reactions [18, 19], and recent reports suggest that GSTP1 can catalyze protein S-glutathionylation via the sulfenic acid intermediate during oxidative stress [20]. Our group further demonstrated that Fas-SSG was preceded by Fas-SOH, and Fas readily interacted with GSTP1 predominantly in the ER within 10 min of stimulation of with FasL [12].

To address the functional importance of ERp57, GSTP and Fas in oxidative processing, S-glutathionylation of Fas, and effects on epithelial cell apoptosis, ERp57 and GSTP1 were individually or simultaneously ablated using an siRNA approach or pharmacologically inhibited. While cells lacking ERp57 showed an almost complete loss of Fas-SSG, and a smaller yet consistent decrease following siRNA-mediated knockdown of GSTP1 upon Fas ligation, simultaneous ablation of both ERp57 and GSTP1 resulted in a complete loss of detectable Fas-SSG in response to FasL. Additionally, siRNA-based ablation of ERp57 and GSTP1 resulted in further decreased caspase-3 and -8 activities and rescued cells from FasL-induced death compared to individual knockdown [12]. Incubation of epithelial cells with thiomuscimol, a known inhibitor of PDIs [21] significantly attenuated Fas-SSG in response to FasL compared to the inactive analog muscimol, with corresponding decreases in caspase-3 and -8 activities. Incubation of cells with TLK199, a highly specific inhibitor of GSTP [22] also resulted in decreased FasL-mediated Fas-SSG, caspase-3, and caspase-8 activities [12]. Taken together, these results demonstrate that the coordinated catalytic activities of ERp57 and GSTP contribute to Fas-SSG and activation of caspases.

Lung fibrosis is believed to be the result of dysregulated epithelial injury/repair mechanisms and aberrant myofibroblast activation and proliferation [23]. However, the mechanistic details whereby oxidative changes intersect with profibrotic signaling pathways remain elusive. Numerous pathways have been linked to the pathogenesis of fibrotic lung disease, including Fas [24, 25]. Immunoprecipitation of Fas from lung tissues of BLM-treated animals showed strong interactions with ERp57 and GSTP1, which were not detected in the PBS control group. In response to siRNA mediated-ablation of ERp57 and GSTP1, mice showed significant decreases in collagen content in lung tissue 15 days following BLM exposure, as well as decreased Fas-SSG and caspase-3 and -8 activities compared to Ctr siRNA-instilled mice [12]. Numerous signaling pathways leading to the progression of apoptosis have been identified. The studies from our laboratories discussed above serve to highlight how changes in the

protein-thiol redox environment influence activation of cell death machinery, advancing our current understanding of cell death mechanisms.

As has been widely discussed in this dissertation, protein S-glutathionylation is increasingly recognized as an important mechanism underlying redox regulation of signaling pathways with downstream effects on numerous cell functions. It is known that S-glutathionylation of ER chaperones such as PDI regulates their function and induction of the unfolded protein response [26, 27], and recently ERp57 was shown to be glutathionylated in LPS-stimulated RAW264.7 mouse macrophages [28]. The evidence presented in this thesis suggests that ER stress, oxidative stress, and inflammatory responses are intimately linked and highlights the potential relevance of investigating the role of the Grx1 and S-glutathionylation of PDIs in epithelial cell ER stress, apoptosis and fibrotic remodeling in allergic asthma.

Implementation: approach for future investigation

The generation of reactive oxygen species is a complex process. The mechanisms of ROS generation and their subsequent impact are likely disease or context dependent. For years, accumulating evidence has clearly demonstrated that ROS are increased in various disease states. These findings imply that administration of antioxidants such as *N*-acetylcysteine or glutathione would counteract these potentially harmful ROS and protect against disease. However, the therapeutic potential of antioxidants in clinical settings remains unsubstantiated [29]. Improved understanding the molecular pathways and targets responsible for oxidative damage may help clarify whether antioxidants are beneficial or harmful towards to treatment or prevention of diseases. A wealth of studies published over the last two decades has demonstrated that oxidants can function as signaling molecules, and are critical regulators of normal cellular processes including differentiation, growth, cell death and senescence [30, 31]. Moreover, the release of oxidants from the mitochondria and other sources, can trigger a protective response that may protect the cell from additional stresses, a process known as hormesis or mitohormesis [32]. It is possible that interruption of this response may in part explain why antioxidants have had limited efficacy in clinical practice. Given this dichotomy regarding the potential effects of ROS, perhaps our investigations should shift away from distinguishing 'bad' from 'good' ROS, and refocus towards determining the extent to which aberrant production of ROS can be dampened, yet homeostatic functions are maintained. These investigations also should include oxidant-induced post-translational

modifications of proteins, and be aimed at maintaining beneficial protein-S-glutathionylation targets, while reversing pathological S-glutathionylation reactions.

Relevance: socioeconomic and environmental importance

The prevalence of asthma varies significantly throughout the world, and the World Health Organization (WHO) estimates the number of asthma patients will increase by 100 million by 2025 [33]. Asthma is more prevalent among working age groups which can negatively impact productivity [34], and coupled with rising costs in patient care, asthma represents a major global economic burden [33]. Additionally, the emergence of new therapies and disease management strategies constitutes a significant cost component in industrialized countries, where medication is generally the largest driver of direct costs of asthma; in comparison, in-patient and out-patient care seem to be the major source of financial burden in developing regions [33]. Evidence suggests that the increasing cost of medication parallels a reduction in the costs and numbers of hospitalizations and patient-care visits [35-37], which may reflect better access to asthma control medications in more affluent countries. However, the landscape of healthcare systems across the world is dynamic, influenced not only by the emergence of new treatments but also evolving genetic, behavioral (e.g., change in smoking rates), and environmental risk factors which all contribute to the economic burden of this disease.

In addition to asthma prevalence, obesity has also dramatically increased in the past two decades and the convergence of these trends has prompted investigations into the relationship between obesity and asthma. A growing body of evidence suggests that obesity is an important risk factor for asthma [38] and prospective studies indicate a probable shared genetic basis for asthma and obesity [39]. Clinical investigations have demonstrated a very complex association between obesity and asthma [40], and suboptimal responses to conventional asthma therapy are often attributed to distinct immunological and physiological phenotypes in overweight patients compared to normal-weight individuals [41]. Some beneficial effects of weight loss on asthma control have been reported [42]; however, additional studies carefully phenotyping matched cohorts of obese and normal-weight subjects with and without asthma are needed to evaluate the impact of obesity on airway physiology and inflammation.

While the cellular and molecular mechanisms underlying the association of obesity and asthma have yet to be fully elucidated, attention is being brought to changes in the redox environment. Increased systemic or airway oxidative stress may be a potential cause for increased severity in the co-

occurrence of obesity and asthma [43]. Supporting this notion, it has been reported that serum levels of GSH are markedly decreased in obese children [44]. Moreover, mice subjected to a high fat diet in the ovalbumin model of allergic airways disease exhibited significantly lower concentrations of GSH in the BALF and lung tissues, accompanied by increased transcriptional activity of NF- κ B compared to non-obese asthmatic mice [45]. These reports provide further rationale to investigate the involvement of the S-glutathionylation/glutaredoxin redox axis in asthma and obesity, and a more thorough understanding of altered redox status will unveil novel mechanisms that can be exploited to develop more targeted therapies, ultimately resulting in better treatment options.

Lastly, it is widely accepted that the global environment of earth is radically shifting, and changes in air quality and climate have a quantifiable impact for both morbidity and mortality of asthma and other respiratory diseases [48]. A statement issued by the European Respiratory Society (ERS) (developed by members of Health and Environmental Network (HENVINET) and the American Thoracic Society) highlights climate change and related health impacts [49], specifically noting altered spatial and temporal distribution of allergens (pollens, molds and mites) [49]. While the effects of climate change on respiratory allergy are not fully known, increased exposure to allergens as a result of global climate change, combined with exposure to pollutants may act synergistically to enhance the allergic response resulting in more prominent and severe disease [50]. While this thesis approaches disease on a cellular and molecular level, the results presented herein add to the collective knowledge of the pathophysiological mechanisms driving allergic asthma.

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